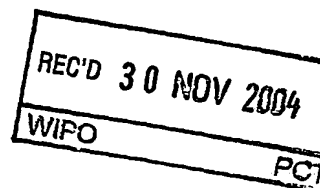




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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03026953.4

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FKBP51 : A novel target for antidepressant therapy

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FKBP51: A Novel Target for Antidepressant Therapy

The present invention relates to a method of classifying an individual comprising analyzing the nucleic acid of a sample taken from said patient for nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 and/or determining the expression level of FKBP51 in said sample. In a preferred embodiment, the invention provides a method of predicting the response to antidepressant therapy. Furthermore, a method of developing an anti-depressant drug, and pharmaceutical compositions are provided.

In this specification, a number of documents is cited. The disclosure of these documents, including manufacturer's manuals, is herewith incorporated by reference in its entirety.

National surveys in Germany and in the United States agree that depression and other affective disorders (e.g. anxiety disorders and bipolar disorder) are among the most common medical conditions worldwide with lifetime prevalence for major depression reaching up to 14%. Pharmacotherapy is an effective treatment of depression and since the serendipitous discovery of the first antidepressant drug imipramine, a number of antidepressant drugs are now available. So far, all commercially available antidepressant drugs share the same pharmacological principle of enhancing monoaminergic neurotransmission, even though this may be achieved through a variety of mechanisms. Despite intensive efforts in the development of antidepressant drugs, major breakthroughs have only been achieved on the side effect profile of these drugs. Even though antidepressants are the most effective treatment for depressive disorders, there is still substantial need for improvement. Adequate therapy response to a single antidepressant is only

observed in 40-60% of patients, even when given in sufficiently high dose for enough time. There is also a substantial lag between the onset of treatment and clinical improvement that can last up to several weeks, even if "therapeutical" plasma concentrations can now be reached in a shorter amount of time. Furthermore, there are a percentage of patients that develop therapy-resistant depression, unresponsive to multiple treatment trials.

Although antidepressant drugs elicit quite divergent immediate effects, it is hypothesized that they all target a common final signaling pathway. The hypothalamic-pituitary-adrenal (HPA) system is considered to play a central pathophysiological role in this disease, because this system is not only consistently dysregulated in patients suffering from major depression, but normalization of this dysregulation is a prerequisite for successful treatment¹. Currently used antidepressant drugs exert instant actions at various elements involved in monoaminergic neurotransmission². These trigger a cascade of events that ultimately lead to the resolution of the clinical symptoms of depression. A host of data implicates central and peripheral disturbances of stress hormone regulation in the pathogenesis of depression and normalization of these defects as a necessary predecessor of clinical response to medication^{3,4}. Central mechanisms for HPA-axis hyperactivity are an increased neurotransmission of the hypothalamic peptides corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) that stimulate adrenocorticotrophic hormone (ACTH) and cortisol release and an impaired negative feedback of this system due to glucocorticoid (GC) receptor (GR) insensitivity^{5,6,7,8}. Preclinical and clinical studies suggest that one mechanism of action of antidepressant drugs may be to restore negative HPA-axis feedback via the GR leading to a downregulation of the overexpressed peptides CRH and AVP^{9,10,11,12,13,14}. For proper functioning the GR, a ligand-activated transcription factor, depends on a large molecular complex that is necessary for proper receptor

transcription and transcriptional regulation of target genes

binding, FKBP51 is replaced by FKBP52, which then recruits dynein into the complex, allowing its nuclear translocation and transcriptional activity²⁰. A naturally occurring overexpression of FKBP51 appears to be the common cause of GR insensitivity in New world monkeys^{21,22,23,26}. Moreover, overexpression of human FKBP51²¹ *in vitro* clearly reduces hormone binding affinity and nuclear translocation of GR. Glucocorticoids induce FKBP51 mRNA expression via an ultra-short negative feedback loop for GR activity²⁴.

It is known that the response to antidepressant therapy varies significantly from patient to patient. So far, these variations can only be determined and analysed in a retrospective manner. However, it would be highly desirable to classify individuals and to predict their response to therapy in order to tailor treatment to the individual patient's specific needs. The technical problem underlying the present invention therefore was to provide methods of classifying individuals.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method of classifying an individual comprising analyzing the nucleic acid of a sample taken from said individual for nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 and/or determining the expression level of FKBP51 in said sample. In other words, said analyzing is performed *in vitro*. In an alternative embodiment, said analyzing is performed *ex vivo* or *in vivo*.

The term "classifying" refers to the assignment of individuals to two or more groups or classes. In other words, individuals, previously unclassified, get labelled by their respective class. The assigned class label may refer to parameters used for classification, e.g. polymorphisms and/or expression levels, or may refer to parameters not used for classification because their values are not known beforehand, e.g. fast or slow response to therapy. In the first case, class discovery methods, e.g. clustering may be applied, whereas in the second case predictive classification methods are used. Classification may be done manually by a trained person or by a computer program provided with the values of the parameters used

for class distinction. Patients have to give informed consent. Data have to be handled and kept secret in accordance with national laws.

The term "nucleotide polymorphism" refers to the occurrence of one or more different nucleotides or bases at a given location on a chromosome. Usually, polymorphisms are distinguished from mutations based on their prevalence. Sometimes a threshold of 1% prevalence in a population of individuals is considered for separating polymorphisms (more frequent) from mutations (less frequent). A single nucleotide polymorphism (SNP) is a polymorphism of a single nucleotide or base. The SNP database maintained at NCBI (<http://www.ncbi.nlm.nih.gov/SNP/>) divides SNPs into SNPs in the proximity of a known locus and such that are 5' further away than 2 kb from the most 5' feature of a gene and 3' further away than 500 bases from the most 3' feature of a gene. SNPs in the proximity of a known locus are further divided into SNPs occurring at an mRNA location and such that do not. SNPs occurring at an mRNA location comprise coding and non-coding SNPs.

In view of the evidence relating to nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 laid down in the present application, the inventors for the first time envisaged classifying individuals based on said polymorphisms.

Nucleotide polymorphisms may be associated or linked to a particular phenotype. Genetic screening exploits this association.

Genetic screening (also called genotyping or molecular screening), can be broadly defined as testing to determine if an individual has mutations, alleles or polymorphisms that either cause a specific phenotype or are "linked" to the mutation causing the phenotype. Linkage refers to the phenomenon that the DNA sequences which are close together in the genome have a tendency to be inherited together.

because, in a given population, they tend to either both occur together or else not occur at all in any particular member of the population. Indeed, where multiple polymorphisms in a given chromosomal region are found to be in linkage disequilibrium with one another, they define a quasi-stable genetic "haplotype", "haplotype block" or "LD block".

Location and size of haplotype blocks can be determined using computer programs, e.g. the program HaploBlockFinder²⁵. Figure 1 shows a linkage disequilibrium map of the FKBP51 region.

Furthermore, where a phenotype-causing polymorphism is found within or in linkage with a haplotype, one or more polymorphic alleles of the haplotype can be used as a diagnostic or prognostic indicator of the likelihood of developing a specific phenotype. Identification of a haplotype which spans or is linked to a phenotype-causing polymorphism, serves as a predictive measure of an individual's likelihood of exhibiting that phenotype-causing polymorphism. Importantly, such prognostic or diagnostic procedures can be utilized without necessitating the identification and isolation of the actual phenotype-causing molecule. This is significant because the precise determination of the molecular basis of the establishment of a specific phenotype can be difficult and laborious, especially in the case of multifactorial phenotype.

Surprisingly, it has been found in healthy individuals that nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 correlate with the expression level of FKBP51. Therefore, according to the invention, the expression level of FKBP51 may be determined instead of or in addition to said nucleotide polymorphisms for the purpose of classifying individuals.

As stated above, there is an unmet need for methods of classifying patients suffering from depression with regard to their response to therapy. Unexpectedly, nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 were found to correlate significantly with the response to antidepressant therapy.

Therefore, it is preferred in accordance with the present invention that said classifying consists of or comprises predicting the response to therapy of an individual, wherein said individual is a patient suffering from depression. The availability of information as to the expected response to therapy is of great advantage for the patient, as a rational choice of drug and/or a tailored therapy regimen minimize adverse side effects and ensure the fastest decline of the symptoms of the disease.

Figure 2 shows a plot of the significance of the association with response to antidepressant therapy of SNPs in or in the proximity of the FKBP51 locus investigated by the inventors.

"Therapy" refers in accordance with the present invention to treatment with antidepressant drugs. Most antidepressant drugs target the transport of the neurotransmitters serotonin and/or norepinephrine, or the activity of the enzyme monoamine oxidase. Common antidepressant drugs include: Selective serotonin-reuptake inhibitors (e.g., fluoxetine, paroxetine, sertraline, fluvoxamine), tricyclic antidepressants (e.g., amitriptyline, imipramine, desipramine, nortriptyline), monoamine oxidase inhibitors (e.g., phenelzine, isocarboxazid, tranylcypromine), and designer antidepressants such as mirtazapine, reboxetine, nefazodone. Benzodiazepines, or lithium carbonate may also be administered.

The inventors surprisingly found that elevated levels of FKBP51 correlate significantly with a faster response to therapy, i.e., the decline of symptoms of depression as determined by the Hamilton Depression Rating Scale (HAM-D) sets in earlier than in patients with lower levels of FKBP51. This observation is indeed unexpected, considering that transcriptional activity of GR heterocomplex requires that FKBP51 dissociates from the heterocomplex. The skilled person would expect that elevated levels of FKBP51 lead to an accumulation of the GR heterocomplex in the cytoplasm,

thereby preventing GR heterocomplexes from entering the nucleus, and consequently preventing GR heterocomplexes from inducing transcription of GR target genes.

FKBP51 mRNA as a marker for the assessment of individual GC sensitivity, for the in vitro measurement of GC potency, and the in vivo determination of GC bioavailability. While Vermeer et al. describe an induced increase of FKBP51 expression, the present invention relates to elevated levels of FKBP51 expression associated with nucleotide polymorphisms. Vermeer et al. do not refer to any polymorphisms in the FKBP51 locus. Furthermore, Vermeer et al. do not refer to any implications of their observations for the field of depression therapy.

Also reference 1 (Holsboer, 2000) discusses the implications of an altered FKBP51 level for corticosteroid signaling. According to Holsboer, ligand-bound GR associates with other proteins comprising heat shock proteins and immunophilins, specifically FKBP51 or FKBP52. An elevated FKBP51 level is discussed in relation to excessively high cortisol levels in squirrel monkeys²⁶, whereby it is speculated that the elevated FKBP51 level serves for compensating the notoriously high cortisol levels in squirrel monkeys.

Holsboer furthermore discusses possible causes of inherited glucocorticoid resistance, viz. a polymorphism in the GR gene or other alterations in genes, whose products are involved in glucocorticoid signaling. However, Holsboer does not designate any specific protein involved in glucocorticoid signaling.

The application of genotyping in the field of pharmacology gave rise to the fields of pharmacogenetics and, more recently, pharmacogenomics, the latter being used to denote studies correlating drug response with the analysis of multiple genes, e.g. their expression and/or polymorphisms or mutations in said genes. It is envisaged to stratify the population such that groups of individuals are obtained which receive tailored treatments. In order to get approval for tailored drugs and/or treatment regimens, the stratification of the population has to be extended to clinical trials.

Therefore, in a preferred embodiment, classifying according to the present invention consists of or comprises selecting an individual for a clinical trial. Individuals selected for clinical trials in accordance with the present invention may comprise healthy individuals and/or patients suffering from depression.

There are several published patent applications in the field of pharmacogenomics, that describe the use of gene sequence variations including SNPs (WO 00/50639, WO 99/64626) or expression profiling on a large scale for evaluating antidepressant and other drug response in patients. They give detailed technical descriptions on how to use SNPs to assess drug response. However, these documents neither anticipate nor suggest to investigate polymorphisms in the FKBP51 locus or FKBP51 expression levels.

It has been found by the inventors that nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 correlate with the number of a patient's previous episodes of depression. Therefore, said polymorphisms and/or the expression level of FKBP51 can be used as (a) prognostic marker/s for an elevated number of episodes of depression.

Accordingly, in a further preferred embodiment, classifying according to the present invention consists of or comprises predicting the predisposition of an individual for an elevated number of episodes of depression, wherein said individual is a patient suffering from depression.

In a preferred embodiment, the nucleic acid to be analyzed is genomic DNA (gDNA).

In a more preferred embodiment, the haplotype block comprises at least one SNP selected from the group consisting of rs4713916, rs3800372, rs1360780 and rs3800373. According to this embodiment, the identification and analysis of any nucleotide polymorphism in said haplotype block is envisaged.

The identifiers recited above permit ~~retrieval~~ of sequence and other information for the respective SNP in the SNP database maintained at the NCBI (<http://www.ncbi.nlm.nih.gov/SNP/>).

consisting of rs4713916, rs3800372, rs1360780 and rs3800373.

These four SNPs are most significantly associated with improved response to antidepressant treatment. The following table summarises the position of these SNPs on chromosome 6, the region of the FKBP51 locus containing the SNP, nucleotide changes for each genotype, heterozygote frequency and the p-value for an association with fast response to an antidepressant drug.

Table 1

SNP	Position On Chr 6	type	Nucleotide change	Heterozygote frequency	p-value
rs4713916	35716838	Promoter region	G/A	36.6%	5.5×10^{-5}
rs3800372	35702100	Intron 1	T/C	33.0%	5.5×10^{-6}
rs1360780	35654426	Intron 2	C/T	40.1%	1.2×10^{-4}
rs3800373	35589331	3'UTR	A/C	33.0%	2.8×10^{-5}

Following the identification of individuals homozygous for the AA allele of rs4713916, or the CC allele of rs3800372, or the TT allele of rs1360780, or the CC allele of rs3800373, or patients with an increase in the expression levels of FKBP51, a normal prudent physician would recommend prescription or administration of an antidepressant drug. Administration and dosage of antidepressant drugs can vary between patients and are well known in the medical art, see, for example Benkert and Hippus, "Kompendium der Psychiatrischen Pharmakotherapie", Springer Verlag Publishing, 2000; Albers, "Handbook of Psychiatric Drugs: 2001-2002 Edition", Current Clinical Strategies Publishing, 2000. Preferred examples include between 5 mg and 80 mg per day, preferably 20 mg, fluoxetine; between 5 mg and 50 mg per day, preferably 20 mg, paroxetine; between 5 mg and 200 mg per day, preferably 50 mg, sertraline; between 5 mg and 300 mg per day, preferably 100 mg, fluvoxamine; between 5 mg and 100 mg per day, preferably 30 mg, mirtazapine; between 4 mg and 50 mg, preferably 8 mg, reboxetine; between 5 mg and 600 mg per day, preferably 200 mg, nefazodone; between 450 mg and 1800 mg per day, preferably 900 mg, lithium carbonate.

Furthermore, the SNPs listed in Table 1 correlate with the number of previous episodes of depression. This is exemplified by the data shown in Figures 5 (rs1360780) and 7 (rs3800372).

Several methods of analyzing a nucleic acid for nucleotide polymorphisms are known in the art. Accordingly, in a preferred embodiment, analyzing the nucleic acid by the method of the invention comprises (a) a primer extension assay; (b) a differential hybridization assay; and/or (c) an assay which detects allele-specific enzyme cleavage.

The underlying principles and the use of said assays has been described in an article of Asil Memisoglu (www.thebiotechclub.org/industry/emerging/pharmacogenomics.php). Examples for said assays are known by a person skilled in the art.

In a more preferred embodiment, the method according to the invention comprises, prior to analyzing, amplification of at least a portion of said haplotype block. Preferably, said amplification is effected by or said amplification is the polymerase chain reaction (PCR). The principles of and procedures to perform PCR are known in the art.

In a more preferred embodiment, said amplification reaction uses primers which hybridize specifically with a portion of said haplotype block. Means of ensuring specificity of hybridization according to the present invention are known in the art and include stringent hybridization conditions. The term "stringent hybridization conditions", as used in the description of the present invention, is well known to the skilled artisan. Appropriate stringent hybridization conditions for each sequence may be established by a person skilled in the art on well-known parameters such as temperature, composition of the nucleic acid molecules, salt conditions etc.; see, for example, Sambrook et al., "Molecular Cloning, A Laboratory Manual"; CSH Press,

citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°. Other stringent hybridization conditions are for example 0.2 x SSC (0.03 M NaCl, 0.003M Natriumcitrat, pH 7) at 65°C.

In a preferred embodiment, the primers to be used for said amplification reaction have the sequence as set forth in SEQ ID NOs: 1 and 2; 3 and 4; 5 and 6; or 7 and 8 and disclosed in the following table.

Table 2

SNP-ID	sense	antisense
rs4713916	ACGTTGGATGTATCTGGCAACCCTAACCTC (SEQ ID NO: 1)	ACGTTGGATGCCTAACGAGATAGTGAGGAG (SEQ ID NO: 2)
rs3800372	ACGTTGGATGAACACCAAGAGAAGCAGCTC (SEQ ID NO: 3)	ACGTTGGATGCTATCCACCTCCTCCATAAG (SEQ ID NO: 4)
rs1360780	ACGTTGGATGAAGAGATCCAGGCACAGAAG (SEQ ID NO: 5)	ACGTTGGATGTGCCAGCAGTAGCAAGTAAG (SEQ ID NO: 6)
rs3800373	ACGTTGGATGAAACCCCTAGTGTAGAAGAG (SEQ ID NO: 7)	ACGTTGGATGTTTACACTCCTCTATCATGC (SEQ ID NO: 8)

In the primer extension assay according to the invention the target sequence is annealed to a primer complementary to the region adjacent to the SNP site. Dideoxyribonucleotides (ddNTPS) and DNA polymerase are added to the mixture and the primer is extended by a single nucleotide. The single nucleotide added is dependent on the allele of the amplified DNA. Primer extension biochemistry can be coupled with a variety of detection schemes, comprising fluorescence, fluorescence polarization (FP), luminescence and mass spectrometry (MS).

Accordingly, in a preferred embodiment of the method of the invention, the primer extension assay uses a primer which hybridizes specifically with a portion of said haplotype block which is adjacent to a polymorphism. The following table discloses the primers used for genotyping using single-base extension assays.

Table 3

SNP-ID	Extension primer
rs4713916	GACTCCTACATTTTCCTCT (SEQ ID NO : 9)
rs3800372	GAAGCAGCTCCCTTTAGA (SEQ ID NO : 10)
rs1360780	GGCTTTCACATAAGCAAAGTTA (SEQ ID NO : 11)
rs3800373	AAGAGCAACTATTTATTGTCAAC (SEQ ID NO : 12)

Therefore, in a preferred embodiment of the method of the invention, the primer to be used for said primer extension assay has the sequence as set forth in SEQ ID NO: 9, 10, 11 or 12.

Further methods of analyzing a nucleic acid for the occurrence of polymorphisms are differential hybridization and allele-specific enzyme cleavage. The latter is also referred to as Restriction Fragment Length Polymorphism (RFLP).

Restriction Fragment Length Polymorphism (RFLP) is a technique in which species, strains, or, as envisaged by the inventors, polymorphic alleles may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme.

In a preferred embodiment, the differential hybridization assay according to the invention or said assay detecting allele-specific enzyme cleavage according to the invention uses probes which hybridize specifically with a portion of said haplotype block which comprises a polymorphism. The detection of allele-specific enzyme

In a more preferred embodiment, said probes have the sequence as set forth in SEQ ID NO: 13 and 14; 15 and 16; 17 and 18; or 19 and 20. The sequences are shown in the table below.

Table 4

SNP	Column A Probe for allele associated with improved response when present in a homozygous state	Column B Probe for allele associated with inferior response when present in a patient
rs4713916	ATTTTCCTCTATCTTGGTCCA (SEQ ID NO: 13)	ATTTTCCTCTGTCTTGGTCCA (SEQ ID NO: 14)
rs3800372	TAAAGTAATTCTCTAAAGGGA (SEQ ID NO: 15)	TAAAGTAATTTCTAAAGGGA (SEQ ID NO: 16)
rs1360780	AGCAAAGTTATACAAAACAAA (SEQ ID NO: 17)	AGCAAAGTTACACAAAACAAA (SEQ ID NO: 18)
rs3800373	ATTTGTCAACCCTACAGATTT (SEQ ID NO: 19)	ATTTGTCAACACTACAGATTT (SEQ ID NO: 20)

Each of these probes recognizes one of the two alleles at the respective SNPs only. For example, in a hybridization experiment for the rs4713916 SNP, a hybridization signal with the probe in column A and no signal with the probe in column B would be indicative of a person with improved response to therapy, whilst a signal with both probes and with only the probe from column B would be indicative of inferior response. The same also applies to the other SNPs (rs3800372, rs1360780, and rs3800373).

In a more preferred embodiment, the probes used for the differential hybridization assay are immobilized on a supporting material. Probe sets, immobilized on a filter or solid support and arranged in arrayed form, are generally referred to as microarrays or DNA chips.

As described above, SNPs which correlate with response to therapy in patients suffering from depression, correlate also with FKBP51 expression levels. The latter correlation has been observed in healthy individuals. Therefore, the administration of antidepressant drugs does not account for the observed differences in FKBP51 expression level. Without being bound by a specific theory, it is assumed that SNPs in the FKBP51 locus are responsible for different FKBP51 expression levels. Therefore, the use of FKBP51 expression levels for classification and prognostic

methods according to the invention is envisaged.

In a preferred embodiment, the FKBP51 expression level to be determined is the mRNA expression level. Methods for the determination of mRNA expression levels are known in the art and comprise Real Time RT-PCR, Northern blotting and hybridization on microarrays or DNA chips equipped with one or more probes or probe sets specific for FKBP51 transcripts.

In a further preferred embodiment, the expression level to be determined is the protein expression level. The skilled person is aware of methods for the quantitation of proteins. Amounts of purified protein in solution can be determined by physical methods, e.g. photometry. Methods of quantifying a particular protein in a mixture rely on specific binding, e.g. of antibodies. Specific detection and quantitation methods exploiting the specificity of antibodies comprise immunohistochemistry (in situ) and surface plasmon resonance. Western blotting combines separation of a mixture of proteins by electrophoresis and specific detection with antibodies. Example 1, sub-heading "Quantification of FKBP51 protein levels in lymphocytes", provides an example of how protein expression levels can be determined according to the invention. Example 4 and Figure 9 present and discuss results obtained by Western blotting and their implications for the methods according to the invention.

In a preferred embodiment, the method of selecting an individual for a clinical trial according to the invention, further comprises the steps: (a) identifying a compound modulating the activity of FKBP51; and optionally (b) optimizing the pharmacological properties of the compound identified in (a); and (c) performing said clinical trials; wherein said clinical trials are clinical trials of the compound identified in (a) or optimized in (b). In this embodiment, the method of the invention may be used for the development of an antidepressant drug.

activator of FKBP51.

In a more preferred embodiment, the compound modulating the activity of FKBP51 increases the expression of FKBP51. The screen described below exemplifies the method for the identification of such compounds.

Screen for compounds that alter the promoter activity of FKBP51

The screen may employ any of the assays described below.

Reporter gene assays employing transient transfection into various cells from yeast to mammals is a standard procedure. For FKBP51, the regulatory regions, i.e. gene promoter and enhancer have to be cloned upstream of the structural part of a gene encoding for a reporter (cf. e.g. Mühlhardt, Der Experimentator: Molekularbiologie, Gustav Fischer Verlag 1999), for example, β -galactosidase, firefly luciferase, renilla luciferase, a fluorescing protein (e.g. GFP, EGFP, EYFP etc.), human growth hormone, CAT (chloramphenicolacetyltransferase), TAT (tyrosylaminotransferase), alkaline phosphatase including SEAP, and peroxidase. Suitable eukaryotic cell lines include immortalized tumour cell lines, listed for example in the catalogues of ATCC and ETCC, in particular the cell lines HeLa, HEK, L929, NIH3T3, COS1, COS7, HepG2, H4-II-E-C3, Saos, K562, SK-N-MC, HT22, CV-1 preferably SK-N-MC or HEK cells. As an alternative to transient transfection assays, an in vitro transcription/translation system may be adapted, or stably transfected cell lines may be used accordingly. A number of methods are established to generate cell lines stably transfected with a reporter gene plasmid.

All assays have to be performed in the presence or absence of test compounds that comprise small molecules, peptides, aptamers and antibodies or fragments or derivatives thereof. An increase in expression of FKBP51 in the presence of a test compound identifies the compound as a hit.

This screen can be extended to modulators of the expression of FKBP52 by replacing the FKBP51 nucleic acid or amino acid sequence with the FKBP52 nucleic acid or amino acid sequence.

In a further preferred embodiment, the compound modulating the activity of FKBP51 enhances function and/or stability of FKBP51. The screen described below exemplifies the method for the identification of such compounds.

Screen for compounds that bind to FKBP51 and change its function or stability

This screen is designed to first identify compounds that bind to FKBP51 and subsequently determine FKBP51-binding compounds that in addition have functional effects.

For the purpose of identifying FKBP51-binding compounds, for example a phage display library may be used. While using a phage display library allows to screen peptide compounds, the Biacore 3000 system is suitable for detection of any compound that binds to FKBP51 larger than 180 dalton. The Biacore system requires purified, human FKBP51, which can be from any source, preferably expressed in a bacterial expression system, preferably as a His-tagged protein. Purification of His-tagged protein is an established, standardized procedure and can be performed according to the manufacturers recommendation, e.g. Qiagen or Invitrogen. Any expression vector for recombinant proteins in bacteria can be used, preferably one that provides inducible expression, preferably the vector series pProExHTa (Invitrogen).

Any technique that attaches the purified FKBP51 protein to the Biacore sensor chip surface may be used. In particular, the use of NTA sensor chips is suitable for the His-tagged version of the recombinant FKBP51. Automatic screening of a large number of compounds can be performed according to established protocols by the Biacore manufacturer. Compounds that bind to FKBP51 will then be subjected to subsequent analyses to identify those with an effect on protein stability of FKBP51, on hormone binding of GR or on GR-dependent transcription.

western blotting techniques on an Imaging System, preferably an imaging station like Kodak 440CF.

To determine hormone binding affinity, a number of different protocols are available. Binding affinity can be measured in tissues, in cell extracts, or in a partially purified extract of the glucocorticoid receptor, which needs to be supplemented with recombinant FKBP51. A recombinant, partially purified human glucocorticoid receptor expressed in the Baculovirus system is available from Panvera.

Assays for determining glucocorticoid receptor-dependent gene transcription are well known in the art, see for example Herr et al., *Mol. Pharmacol.* 57 (2000), 732-737; Hollenberg et al., *Cancer Res.* 49 (1989), 2292s-2294s; Kullmann et al., *J. Biol. Chem.* 273 (1998), 14620-14625; Rupprecht et al., *Eur. J. Pharmacol.* 247 (1993), 145-154. These assays may be performed using one of the transient transfection methods or in stably transfected cell lines.

This screen can be extended to FKBP52 binders and modulators by replacing the FKBP51 nucleic acid or amino acid sequence with the FKBP52 nucleic acid or amino acid sequence.

Methods for the optimization of the pharmacological properties of compounds identified in screens, generally referred to as lead compounds, are known in the art and comprise a method of modifying a compound identified as a lead compound to achieve: (i) modified site of action, spectrum of activity, organ specificity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of therapeutic action, duration of effect, and/or (vi) modified pharmacokinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically

acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophilic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl group to ketals, acetals, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetals, ketals, enolesters, oxazolidines, thiozolidines or combinations thereof; said method optionally further comprising the steps of the above described methods.

The various steps recited above are generally known in the art. They include or rely on quantitative structure-action relationship (QSAR) analyses (Kubinyi, "Hansch-Analysis and Related Approaches", VCH Verlag, Weinheim, 1992), combinatorial biochemistry, classical chemistry and others (see, for example, Holzgrabe and Bechtold, Deutsche Apotheker Zeitung 140(8), 813-823, 2000).

Clinical trials according to the invention can for example be performed as described in the following protocol.

Protocols for clinical trials

Response to treatment by an antidepressant, time until response (i.e. latency) to an antidepressant, or patient outcome to an antidepressant treatment protocol can be predicted by determining in patients the presence of at least one of the FKBP51 SNPs disclosed in Table 1. Accordingly, ~~an individual homozygous for the AA allele of rs4713916, or the CC allele of rs3800372, or the TT allele of rs1360780, or the CC allele of rs3800373, or with elevated FKBP51 expression levels would have an~~

~~increased risk of relapse to depression after treatment with an antidepressant.~~

FKBP51 expression would have an inferior, or slower response to an antidepressant drug. The various alleles are summarised in the following table.

Table 5

SNP	Column A Allele associated with improved response	Column B Alleles associated with inferior response
rs4713916	AA	GG or AG
rs3800372	CC	TT or TC
rs1360780	TT	CC or CT
rs3800373	CC	AA or AC

In a clinical trial for a new antidepressant drugs, patients would be grouped or selected according to the presence of one or more of the alleles found in column A. In addition, patients could be selected according to elevated FKBP51 levels. Such patients would then be enrolled into a clinical trial for testing the efficacy of a new antidepressant drug where the endpoint would be improvement in the Hamilton Depression Rating score, dexamethasone suppression test, or combined dexamethasone - corticotropin releasing hormone test. Results obtained with this group of patients could be compared to a second group of patients which possess one or more of the alleles found in column B or a group of patients with low FKBP51 expression.

In a more preferred embodiment, the method of classifying patients suffering from depression further comprises the step of treating said patient with an antidepressant.

In view of the favourable response to therapy associated with elevated levels of FKBP51, the invention also provides a pharmaceutical composition comprising (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451; (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having the biological activity of the polypeptide with the sequence of SwissProt accession number Q13451; and/or (c) a polypeptide encoded by the nucleic acid of (a) or (b).

Said nucleic acids are provided in a formulation and to be administered via a route which ensures their expression.

Preferably, said hybridizing occurs under stringent conditions.

SwissProt (<http://us.expasy.org/>) is a manually curated database of annotated protein sequences. The recited identifier (Q13451) refers to the SwissProt entry for human FK506-binding protein 51 (FKBP51), sometimes also referred to as FKBP5, FKBP54 or AIG6. FKBP51 acts as a peptidyl-prolyl cis-trans isomerase (rotamase).

The invention also relates to the use of (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451; (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having the biological activity of the polypeptide with the sequence of SwissProt accession number Q13451; (c) a polypeptide encoded by the nucleic acid of (a) or (b); (d) an activator of the expression of the polypeptide with the sequence of SwissProt accession number Q13451; and/or (e) an activator of the polypeptide with the sequence of SwissProt accession number Q13451 for the manufacture of a pharmaceutical composition for the treatment of depression. Said activator of expression can be obtained by the above described screen for compounds that alter the promoter activity of FKBP51. Said activator of the polypeptide can be identified by the above described screen for compounds that bind FKBP51 and change its function or stability.

Furthermore, the invention provides a method of treating depression comprising the administration of (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451; (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having

depression.

Recent studies have shown that FKBP51 and FKBP52 are physiologically relevant regulators of glucocorticoid receptor (GR) sensitivity. In the absence of steroids, FKBP51 interacts with the GR complex to retain it in the cytoplasm where it is inactive. Upon steroid binding, FKBP51 is replaced by FKBP52 which results in a recruitment of dynein into the GR complex, allowing nuclear translocation of the complex and transcriptional activity of the glucocorticoid receptor (Davies et al., J. Biol. Chem. 227 (2002), 4597-4600).

Thus, inhibitors of FKBP52 may have antidepressant effects by allowing FKBP51 to remain in contact with the GR complex and keeping the glucocorticoid receptor in an cytoplasmic inactive form despite steroid binding. The term "inhibitor of FKBP52" refers to substances inhibiting the biochemical or catalytic activity and/or the biological activity of FKBP52. The latter class of substances comprises compounds interfering with the interaction between FKBP52 and its interaction partners. Said interaction partners comprise components of the GR complex, including heat shock proteins such as Hsp90.

There are several examples of known or recognised FKBP52 inhibitors described in the art. Furthermore, inhibitors of immunophilins (or peptidyl-prolyl cis-transisomerases or rotamases) in general or of any specific immunophilin other than FKBP52 is a potential inhibitor of FKBP52. Whether such substance does inhibit FKBP52 can be assayed with the screens or assays described above.

A screening method to identify inhibitors of FKBP52 binding to partner proteins such as Hsp90 can be performed using bioluminescence resonance energy transfer (BRET). Briefly, the FKBP52 protein is fused to Renilla luciferase, whereas the Hsp90 protein is fused to a fluorescent protein, for example the yellow fluorescent protein (YFP). The emission of the luciferase signal is detected following addition of its substrate coelenterazine. When FKBP52 binds to Hsp90, a resonance energy transfer occurs between the luciferase and the YFP resulting in the detection of both the luciferase and the YFP signals. An inhibitor compound preventing binding of FKBP52 to Hsp90 would result in the emission of the luciferase signal only. The use

of BRET for drug screening is well known in the art, see for example Xu, Y. et al. (1999) A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc. Natl. Acad. Sci. U. S. A.* 96: 151-156; Boute et al. (2002) The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol. Sci.* 23:351-354.

Identification of inhibitors of FKBP52 protein binding can also be done using the CheckMate™ Mammalian Two-Hybrid System from Promega or fluorescence resonance energy transfer (FRET). Such methods are well known in the art, see for example Serebriiskii et al. (2002) Detection of peptides, proteins, and drugs that selectively interact with protein targets. *Genome Res.* 12:1785-1791; Bergendahl et al. (2003) Luminescence resonance energy transfer-based high-throughput screening assay for inhibitors of essential protein-protein interactions in bacterial RNA polymerase. *Appl. Environ. Microbiol.* 69:1492-1498.

International Patent Applications WO 98/13343, WO 98/13355, WO 98/20891, WO 98/20892 and WO 98/20893 describe various pyrrolidine, piperidine and homopiperidine derivatives having an acyl, amide, oxalyl, or similar linking group, at the 1-position of the heterocycle.

US 5,721,256 describes various pyrrolidine, piperidine and homopiperidine derivatives having an SO₂ linking group at the 1-position of the heterocycle.

US 6,166,011 and US 6,509,464 disclose compounds that inhibit FKBP12 and FKBP52 without inhibiting the protein phosphatase calcineurin and therefore lack any significant immunosuppressive activity.

WO 99/21552 discloses an FKBP52 antibody and geldanamycin derivatives that can disrupt and inhibit the association between-FKBP52 and Hsp90.

The present invention provides a pharmaceutical composition containing a recognised inhibitor of FKBP52.

treatment of depression. Suitable geldanamycin derivatives or analogs can be identified by the screens described above.

In a preferred embodiment, said inhibitor is an antibody or an aptamer specifically recognizing FKBP52 or a fragment or epitope thereof. Said antibody may be a monoclonal or a polyclonal antibody.

A more preferred embodiment of the invention relates to an antibody which is a monoclonal antibody.

Said antibody, which is monoclonal antibody, polyclonal antibody, single chain antibody, or fragment thereof that specifically binds FKBP52 also includes a bispecific antibody, synthetic antibody, antibody fragment, such as Fab, a $F(ab_2)'$, Fv or scFv fragments etc., or a chemically modified derivative of any of these (all comprised by the term "antibody"). Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, *Nature* 256 (1975), 495, and Galfré, *Meth. Enzymol.* 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals with modifications developed by the art. Furthermore, antibodies or fragments thereof to the FKBP52 can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. When derivatives of said antibodies are obtained by the phage display technique, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of FKBP52 (Schier, *Human Antibodies Hybridomas* 7 (1996), 97-105; Malmberg, *J. Immunol. Methods* 183 (1995), 7-13). The production of chimeric antibodies is described, for example, in WO89/09622. A further source of antibodies to be utilized in accordance with the present invention are so-called xenogenic antibodies. The general principle for the production of xenogenic antibodies such as human antibodies in mice is described in, e.g., WO 91/10741, WO 94/02602, WO 96/34096 and WO 96/33735. Antibodies to be employed in accordance with the invention or their corresponding immunoglobulin chain(s) can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s).

known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook (1989), loc. cit..

The term "monoclonal" or "polyclonal antibody" (see Harlow and Lane, (1988), loc. cit.) also relates to derivatives of said antibodies which retain or essentially retain their binding specificity. Whereas particularly preferred embodiments of said derivatives are specified further herein below, other preferred derivatives of such antibodies are chimeric antibodies comprising, for example, a mouse or rat variable region and a human constant region.

The term "scFv fragment" (single-chain Fv fragment) is well understood in the art and preferred due to its small size and the possibility to recombinantly produce such fragments.

The term "specifically binds" in connection with the antibody used in accordance with the present invention means that the antibody etc. does not or essentially does not cross-react with (poly)peptides of similar structures. Cross-reactivity of a panel of antibodies etc. under investigation may be tested, for example, by assessing binding of said panel of antibodies etc. under conventional conditions (see, e.g., Harlow and Lane, (1988), loc. cit.) to the (poly)peptide of interest as well as to a number of more or less (structurally and/or functionally) closely related (poly)peptides. Only those antibodies that bind to the (poly)peptide/protein of interest but do not or do not essentially bind to any of the other (poly)peptides which are preferably expressed by the same tissue as the (poly)peptide of interest, are considered specific for the (poly)peptide/protein of interest and selected for further studies in accordance with the invention.

The invention also provides a ~~method~~ of treating depression comprising the administration of (a) geldanamycin or a geldanamycin derivative that can disrupt and inhibit the association between FAKBP52 and Hsp90; and/or (b) an antibody or an

The Figures show:

Figure 1. Representation of linkage disequilibrium (LD) structure using D' in the FKBP51 region. The 4 SNPs of the invention are labelled with a red arrow. The quantity D' (D prime) measures the amount of linkage disequilibrium between alleles at two loci. It is defined as the difference between an observed haplotype frequency and its expected value under the assumption of no disequilibrium. This difference is then divided by its theoretical maximum. The range of D' therefore goes from 0, indicating no linkage disequilibrium to 1, indicating maximum disequilibrium. D' is defined and first published by Lewontin in 1964²⁷.

Figure 2. Association of SNPs in the FKBP51 gene region and response to antidepressant drugs at week 2. The $-\log p$ values (y axis) of the association were plotted against the physical location of the SNPs (x axis).

Figure 3. Hamilton Depression Rating Scale (HAM-D) scores over the first 5 weeks of hospitalisation according to the rs1360780 genotype. A repeated measures ANOVA showed an overall significant genotype effect: $F_{197,2} = 5.3$, $p = 0.0058$. Patients did not differ in disease severity at admission: mean HAM-D score at admission (SD) for CC = 26.2 (7.3); CT = 25.7 (8.1); TT = 26.2 (7.2). A one way ANOVA showed a non-significant genotype effect for differences in HAM-D scores at admission: $F_{277,2} = 0.15$, $p = 0.86$.

Figure 4. Effect of rs1360780 genotype on ACTH response in the Dex-CRH test at admission and discharge. A t-test showed that peak ACTH values ($T = 3.48$; $df = 95.8$; $p = 0.00075$) and area under the curve of the ACTH response ($T = 1.92$; $df = 98.1$; $p = 0.056$) were significantly lower in the TT genotype vs. the two other genotypes. The cortisol response at admission and the ACTH and cortisol responses at discharge were not significantly different between these two groups.

Figure 5. Number of previous depressive episodes and rs1360780 genotype.

Figure 6. HAM-D scores over the first 5 weeks of hospitalisation against rs3800372 genotypes. A repeated measures ANOVA showed an overall significant genotype

effect: $F_{197,2} = 4.5$, $p = 0.014$ and interaction between response and genotype: $F_{197,4.5} = 2.6$, $p = 0.03$. Patients did not differ in disease severity at admission: mean HAM-D score at admission (SD) for CC = 26.6 (7.1); TC = 25.7 (8.2); TT = 26.1 (7.4). A one-way ANOVA showed a non-significant genotype effect for differences in HAM-D scores at admission: $F_{280,2} = 0.18$, $p = 0.83$.

Figure 7. Number of previous depressive episodes and rs3800372 genotype. A one way ANOVA showed a significant genotype effect: $F_{258,2} = 4.3$; $p = 0.014$.

Figure 8. Western blot of FKBP51 protein for the various genotype of the rs3800372 SNP.

Figure 9. FKBP51 protein levels for the various genotype of the rs3800372 SNP.

The Examples illustrate the invention.

Example 1

Methods

Patients:

256 patients admitted to our psychiatric hospital for treatment of a depressive disorder presenting with a unipolar depressive episode (86.6%), bipolar disorder (12.0%) or dysthymia (1.2%) as their primary psychiatric diagnoses were recruited for the study. Patients were included in the study within 1-3 days of admission to our hospital and the diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Depressive disorders due to a medical or neurological condition were exclusion criteria. Ethnicity was recorded using a self-report sheet for perceived nationality, mother language and ethnicity of the subject itself and all 4 grandparents. All included patients were Caucasian and 92 % of German origin. The study has been approved by the local ethics committee. Written informed consent was obtained from all subjects. Severity of psychopathology at admission was assessed using the 21 items Hamilton Depression Rating Scale (HAM-D) by trained raters, including residents in psychiatry and psychologists. Ratings were performed within 3 days of admission and then in weekly intervals until discharge.

DNA preparation:

On enrollment in the study, 40 ml of EDTA blood were drawn from each patient and DNA was extracted from fresh blood using the Puregene® whole blood DNA-extraction kit (Gentra Systems Inc; MN).

Genotyping:

86 SNPs in the FKBP51 locus were selected from the public SNP database dbSNP (<http://www.ncbi.nlm.nih.gov/80/>). Prior to genotyping, the relevant regions were amplified by PCR. Table 6 shows the PCR primers used. The four SNPs showing the most significant correlation with antidepressant response are shown in bold. Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray®

system) employing the Spectrodesigner software (SequenomTM; CA) for primer selection and multiplexing and the homogeneous mass-extension (hMe) process for producing primer extension products. Table 7 shows the primers used for the primer extension assays. Again, The four SNPs showing the most significant correlation with antidepressant response are shown in bold.

Table 6

SNP-ID	sense	antisense
rs1334894	ACGTTGGATGCTTGTTCATCCCAGCATCTTC	ACGTTGGATGCCCTATTTGGCCTCAAAAAG
rs1360780	ACGTTGGATGAAGAGATCCAGGCACAGAAG	ACGTTGGATGTGCCAGCAGTAGCAAGTAAG
rs15258	ACGTTGGATGGTCAGTATGGCAAATGAGTG	ACGTTGGATGTTTTTCAGCCCGGGTATAAGC
rs1546428	ACGTTGGATGAAACGGAAGCAGAGTCACTC	ACGTTGGATGGGCTGAAAAATCCGGAAAAAG
rs1802039	ACGTTGGATGGAGGTGTAGCAGAAAAAGGC	ACGTTGGATGTCAGGTAAATTCGCTCCAG
rs1803817	ACGTTGGATGTGGAGAGGGGCTATGCTTCTG	ACGTTGGATGCACAGAGATGAAGGAGGAGC
rs1903370	ACGTTGGATGCATTGTGTATCCTTCCAGGC	ACGTTGGATGAGGGACTACAAATCAGTCTG
rs1981656	ACGTTGGATGTACTCACTTTCTCCCCCTTC	ACGTTGGATGTAGTGGACAAAGACTCGGTC
rs2118727	ACGTTGGATGAGCTCCAAAGTATGCTACCG	ACGTTGGATGTCTAGCCCACAGTTGTGATG
rs2290893	ACGTTGGATGAGCCTTCTCCAAGATAGGTC	ACGTTGGATGGCTCTTCAGCGATTTTCGTAG
rs258813	ACGTTGGATGTTGATCATGGGAAGACATGG	ACGTTGGATGTAGGATGCGCCTTTTTCTCC
rs2740204	ACGTTGGATGTGTGTTTCTCTGGGTGTCTG	ACGTTGGATGAACTGCTAAGAACGTGTCCG
rs2958153	ACGTTGGATGAAGGCCTAGGGTGAAGTTAC	ACGTTGGATGCCTACTTTTACCCCTTTGGC
rs2958154	ACGTTGGATGCCAGCATCACTACATGATAG	ACGTTGGATGGCATGTTTCAGAGTCCAGAG
rs3021522	ACGTTGGATGACCCAGGAGAGAAACAAAG	ACGTTGGATGCAGGGTCTTCCCATCTAAAG
rs3176921	ACGTTGGATGTATGAGCCTGCAGAAGCAAG	ACGTTGGATGTGGGAGTAGCTCTTGTCATC
rs3204090	ACGTTGGATGTCCCCAACTTGGCTATGAAAG	ACGTTGGATGAGGGAACCTCAGTAGTCCTC
rs33388	ACGTTGGATGTCTGCCTGAATGAATGGGTG	ACGTTGGATGTGGGTGAAAGTCATGGATGG
rs33389	ACGTTGGATGTCTGCCTGAATGAATGGGTG	ACGTTGGATGTGGGTGAAAGTCATGGATGG

rs6188	ACGTTGGATGTAAACTGTGTAGCGCAGACC	ACGTTGGATGTGTAGTGGCCTGCTGAATTC
rs6191	ACGTTGGATGTTTCCATCTTGGCTGGTCAC	ACGTTGGATGCCTTCTGACACTAAAACCAG
rs6192	ACGTTGGATGCCAAGCAGCGAAGACTTTTG	ACGTTGGATGACTGCTTTGGACAGATCTGG
rs6195	ACGTTGGATGCATTCCACCAATTCCCGTTG	ACGTTGGATGTCCCCAGAGAAGTCAAGTTG
rs6196	ACGTTGGATGGGCAGTCACTTTTGATGAAAC	ACGTTGGATGCCGAGATGTTAGCTGAAATC
rs6597	ACGTTGGATGCTGGCAAGCAGGAAATGTGG	ACGTTGGATGAGATGGACCTGGCCAGATGTC
rs706118	ACGTTGGATGAATGTCTGGGCCATAGGAAC	ACGTTGGATGTTTCCTGTCACAATGAGCCC
rs706120	ACGTTGGATGAGACTGGAACCTCAGGACTTG	ACGTTGGATGTAGGTCTAGCAGCTTTCTGC
rs734369	ACGTTGGATGATAAGGCTGCAAGACTGCAG	ACGTTGGATGATGTTGGCGTATATCCTGCG
rs737054	ACGTTGGATGACAAGGTGACCGAGAACATG	ACGTTGGATGGGTATATTTGGTCAGGTGCC
rs755658	ACGTTGGATGACTAGGATTTACCACAGCCC	ACGTTGGATGGGTGAGGGTTGGAGTATTTG
rs2103681	ACGTTGGATGCTTGAGCTGACATGAGCATC	ACGTTGGATGAACAGCTGACCCACAGAATC
rs1883636	ACGTTGGATGAGTCTTGAGGGTTCACTCAG	ACGTTGGATGTCTTACTGGGCCCCAAACAAG
rs1540910	ACGTTGGATGTGCTCCAAAGTCCCTATTCC	ACGTTGGATGTTGCATGTCTGGAGATCTGG
rs4713878	ACGTTGGATGCTAAACACTGGCTGTGTGAC	ACGTTGGATGTTTCATCCTCACAGCACACTC
rs1883637	ACGTTGGATGAGAAGCTGGGCAGATTTCTC	ACGTTGGATGGGCCCTGAATCAGTCTTAAG
rs992105	ACGTTGGATGGGCATGGCCTTAACCTTTGTG	ACGTTGGATGGAACGTACTCTGGTAAGCAC
rs1051952	ACGTTGGATGAACATCTGGAGTTGGAGCCG	ACGTTGGATGGGGAGAGGGGAGGTTAAA
rs2273000	ACGTTGGATGTAGAATCCTCTGTCTCCTC	ACGTTGGATGAATTTCTCCCCACGATGGTC
rs4713897	ACGTTGGATGGCCCTGAGTATACTTTCTGG	ACGTTGGATGCACTGGGCATTTGCCCATTT
rs734369	ACGTTGGATGATAAGGCTGCAAGACTGCAG	ACGTTGGATGATGTTGGCGTATATCCTGCG
rs747411	ACGTTGGATGGACGTGACACCACACTTGAC	ACGTTGGATGAGGCAGGAGGATCTCTTGAG
rs1320991	ACGTTGGATGGTGTGCGTATGCATACTGTG	ACGTTGGATGTGCCTGCACTGAAAACATGC
rs737054	ACGTTGGATGACAAGGTGACCGAGAACATG	ACGTTGGATGGGTATATTTGGTCAGGTGCC
rs3777747	ACGTTGGATGCCACTCTTACATTCCTCTCC	ACGTTGGATGTCCCTCTCCAAATCTCACTG
rs4401662	ACGTTGGATGAATTGCTTGAACCCAGGAGG	ACGTTGGATGAGTCTTGCTCTGTCAATCCAG
rs4713908	ACGTTGGATGATTCCGTTGTGTGTGTATGC	ACGTTGGATGTCAGACATCTGTCAATCTTC
rs755658	ACGTTGGATGACTAGGATTTACCACAGCCC	ACGTTGGATGGGTGAGGGTTGGAGTATTTG
rs4713899	ACGTTGGATGAGTGGAGCTATAGGAGCTAG	ACGTTGGATGACGGAAAGACTGCTGATTGC
rs1360780	ACGTTGGATGAAGAGATCCAGGCACAGAAG	ACGTTGGATGTGCCAGCAGTAGCAAGTAAG
rs4713907	ACGTTGGATGTCTTGACTTTGTGATCCACC	ACGTTGGATGATACCATACTCTAGGCTGGG
rs3800372	ACGTTGGATGAACACCAAGAGAAGCAGCTC	ACGTTGGATGCTATCCACCTCCTCCATAAG

rs2092427	ACGTTGGATGACCTGCAGTACTTTTGGCAG	ACGTTGGATGCAGACACTTTCTAAGTGCTG
rs4713905	ACGTTGGATGTTCAATACCTCACCTGTCTC	ACGTTGGATGGTGTATTCTGCTTGTATTGGG
rs4520009	ACGTTGGATGACGAACAGGAACTGAAAGC	ACGTTGGATGGAGCATGGTTTTCAGTAAAG
rs4713906	ACGTTGGATGAAGTATTTGTGGCTGGAGGC	ACGTTGGATGATCTCCTGACCTTGTGATCC
rs4713916	ACGTTGGATGTATCTGGCAACCCTAACCTC	ACGTTGGATGCCTAACGAGATAGTGAGGAG
rs2143404	ACGTTGGATGGGTTAGGTAGAGCTCAGTTC	ACGTTGGATGGTAGAGAACCTGGTAAGAAG
rs4713902	ACGTTGGATGGGAGCCAAAACATGAAGAGC	ACGTTGGATGTAGGCAACCTGTATAAGCTG
rs1334894	ACGTTGGATGCTTGTCTATCCCAGCATCTTC	ACGTTGGATGCCCTATTTGGCCTCAAAAAG
rs1475774	ACGTTGGATGCAAGTGAAAACTCCACACC	ACGTTGGATGCCATAAGTCTTTGTCCACAAG
rs2817035	ACGTTGGATGCCTCTTTCTCCTAGGATCC	ACGTTGGATGGTTGCAACAGAGGTAGGAG
rs3807050	ACGTTGGATGAGAGGGAGGGAATAGTTCAG	ACGTTGGATGTGTGTCTCCAAGACTGTGTG
rs1977655	ACGTTGGATGCCCCGTCTCTGCTAAAAATAC	ACGTTGGATGCCAAGTTCAAGCGATTCTTG
rs3800374	ACGTTGGATGAGACGATGGACCCATTTTAC	ACGTTGGATGTCTTTTCCAAGTGGTGAACC
rs4713912	ACGTTGGATGAGGTTCAAGCGATTCTCCTG	ACGTTGGATGCAAAAATTAGCCGGGCTTGG
rs2817047	ACGTTGGATGTCTGGGCTCAAGTATTCTC	ACGTTGGATGAATTAGCCAGGCATGATGGC
rs4713913	ACGTTGGATGGAATTTAACTAGGAGTGCTG	ACGTTGGATGAGCGAGACTCCGTCTCAAAA
rs4713921	ACGTTGGATGAAGCCCTGTGGTTTTATGCC	ACGTTGGATGTGGAACAATTCTGTCCCCAG
rs2817041	ACGTTGGATGGTACACACAGCGAGTGATAC	ACGTTGGATGCTCCTCAACTCTTTGGAGTG
rs2766543	ACGTTGGATGTCTGTGGTTGGCCTTTTCTC	ACGTTGGATGAAGCAGAGCTGCCCCAATAAG
rs5020575	ACGTTGGATGTGATCTTGGCTCACTGCAAC	ACGTTGGATGAAATTAGCCAAGCGTGGTG
rs1591365	ACGTTGGATGGTGGCAAATAGGAGTTCTCC	ACGTTGGATGTTGGCAGGTGTTTTTCTGAG
rs2296662	ACGTTGGATGAATTGGCCTATGACCAGCAC	ACGTTGGATGAAGAGGAGGAGAAACCAGAG
rs2817010	ACGTTGGATGTGGTGTGTATGAGAAGCAGC	ACGTTGGATGTAAGTCTGTGCAGACGGTGG
rs873941	ACGTTGGATGGGGTGATGCTTTTGCAACTC	ACGTTGGATGGCTATGATACCTGGCTGATC
rs3800373	ACGTTGGATGAAACCCCTAGTGTAGAAGAG	ACGTTGGATGTTTACACTCCTCTATCATGC
rs4713903	ACGTTGGATGGAGTCTAAACAAAAACATCC	ACGTTGGATGAAGTACTGGGATTACAGGTG
rs2766534	ACGTTGGATGTCTGAGGGGCTTATTCCTCC	ACGTTGGATGTCTGAGGAGGAGGCTCTCAG

SNP-ID	extension primer
rs1334894	TTGATGACTTAGTCCTGTC
rs1360780	GGCTTTCACATAAGCAAAGTTA
rs15258	TTTAATACACTATTGGATTTTTT
rs1546428	AATATGCCTCCTGGCGTT
rs1802039	AGCAGAAAAAGGCTGTGCTGCC
rs1803817	CTATGCTTCTGTCTCCAC
rs1903370	TCCTTCCAGGCTTTTCTTTAG
rs1981656	CAAGAGAGAGGGCACAGGT
rs2118727	AAAGTATGCTACCGGAAACAC
rs2290893	GTAAATTATGAGACAACTTTT
rs258813	CTAACTACAGTGATTTTGTC
rs2740204	TGGGTGTCTGTGGCTCT
rs2958153	GTTACCGAAAGAGGCGAGTA
rs2958154	AGCTTGTCATTTCTCACCTTT
rs3021522	GAGTCACGTACAGGGTG
rs3176921	AGCCTGCAGAAGCAAGGCCAATAA
rs3204090	GTTATTGACGCATTCATCTCTGA
rs33388	ATGCTTCTCTAGGTGTGTGA
rs33391	CCTCTGAAAATCCTGGTAA
rs5195	GCTGCCAGGAGGAGAACTACCTGC
rs6156	GGAAGACAACCTCCAGAG
rs6157	CAGAGAGACGTCTCCGG
rs6158	GCGCTTCGCAGGTGAGC
rs6159	CTCCTCGCTCCTCGCCGG
rs6188	TTACAGTTCATTTCTATGTATTT
rs6191	CTGTAGGTGAATGTGTTTTT
rs6192	GCGAAGACTTTTGGTTGAT
rs6195	TTCCCGTTGGTTCCGAAA
rs6196	ACAGAAGTTTTTTGATATTTC
rs6597	TGGGGAAGTGTGGATGTTAGC

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rs4713902	CATGAAGAGCTAATTCCTTTATCA
rs1334894	GGAAGTTGATGACTTAGTCCTGTC
rs1475774	CTTGTTTTCTAATGATTCAAG
rs2817035	GGCTCCCTACTCCACCACTAC
rs3807050	CCAGTAAAGACTTGCCTGA
rs1977655	TCTGCTAAAAATACAAAAATAGCC
rs3800374	CCCATTTTACAGTTATGGGCTC
rs4713912	GCCACCCGAGAAGCTGGGATTA
rs2817047	CCCCCAAGTAGCTGGGACTACA
rs4713913	GTGTTTTTGTTTGTTGTTGTTT
rs4713921	CAGTTGAAAGAGCCTCAC
rs2817041	GTGCAGGATCATGCTCTTGGC
rs2766543	CTTTTCTCCCTGCTACGT
rs5020575	AGTGATTCTCGTGCCTCA
rs1591365	TGTCAGTTGTTTTTCCTTGAA
rs2296662	CTGGGCTCGCTTCCCTCCC
rs2817010	AGAAGCAGCCTATGTTGAGG
rs873941	TTTGTCAACTCACTTTTAAAA
rs3800373	AAGAGCAACTATTTATTTGTCAAC
rs4713903	CCAAACAATAAATTGGGAAA
rs2766534	CCGCCCTACACTTTCAC
rs2766554	CTTTTGCCCTCACATTCTT
rs2817054	AGCAGCGGCTCAGGCAG
rs2766597	AGAAGATCCTGATCCTCC

Neuroendocrine assessment using the combined dexamethasone suppression/CRH stimulation (Dex-CRH) test:

The Dex-CRH test was performed as described in detail in Heuser et al., 1994¹⁸. Patients were administered the test within the first ten days of admission (n= 225) and the last ten days of discharge (n = 150). Briefly, patients were pre-treated with 1.5 mg of dexamethasone per os at 23:00. The following day a venous catheter was

placed at 14:30 and blood was drawn at 15:00, 15:30, 15:45, 16:00 and 16:15 into tubes containing EDTA and trasylol (Bayer Inc., Germany). At 15:02 100µg of human CRH (Ferring Inc., Kiel, Germany) was administered intravenously. For the area under the curve (AUC) of the cortisol and ACTH response, the area under the concentration-time course curve corrected for the baseline value at 15:00 was computed using a trapezoidal integration for the test at admission as well as at discharge. Hormone assays for the Dex-CRH test were identical to those described in detail in Zobel et al., 2001¹⁷.

Quantification of FKBP51 protein levels in lymphocytes:

T cells were extracted from the whole blood of healthy controls using magnetic beads with antibodies against CD2 (Dynal Inc.). Cells were then lysed in a protein lysis buffer. Protein levels of FKBP51 were assessed using Western-blot analysis.

For protein extraction cells were solubilized in 1ml lysis buffer (20 mM Tris/HCl pH 7.5; 130 mM NaCl; 20 mM Na₂MoO₄; 1 mM EDTA; 10% glycerol; 0.5% Triton 100; 1:100 protease inhibitor cocktail P2714 from Sigma). The extract was incubated for 1 h on ice, centrifuged for 4 min at 13000 rpm and protein concentration was determined.

For immunoblot detection 10 µg of cell lysates' total protein were separated by SDS-PAGE under denaturing conditions. The proteins were transferred to polyvinylidene difluoride membrane (Schleicher & Schüll GmbH, Germany). Nonspecific binding to membrane was blocked by 5% nonfat milk in Tris-buffered saline/Tween buffer. FKBP51 was detected by a monoclonal anti-FKBP51 antibody (Stress Gen Biotech) followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (Sigma). Signals were visualized by ECL solution (Amersham Pharmacia Biotech) and monitored in an imaging system (Kodak-Imaging Station 440). Light emission was normalized by the intensity of recombinant FKBP5 standard that was loaded onto each gel to ensure accurate comparison of probes loaded on different gels.

Statistical analysis

Statistical analysis was performed using the Student's t-test. A p-value of less than 0.05 was considered significant. All data are presented as mean ± SEM.

Example 2

Association study of polymorphisms in FKBP51 and response to antidepressant drugs

4 SNPs in FKBP51 (see table 1) were genotyped in 327 patients with major depression. 103 of them showed an improvement of over 25% in disease severity as measured by the Hamilton Depression Rating Scale (HAM-D) after two weeks of treatment with an antidepressant (response), while 156 (non-response) showed less than 25% improvement. The patient population used for the studies comprises of individuals that were treated with various antidepressant drugs including, Amitriptylin, Amitriptylinoxid, Clomipramin, Doxepin, Imipramin, Nortriptylin, Trimipramin, Citalopram, Fluoxetin, Fluvoxamin, Paroxetin, Sertralin, Tianeptin, Mirtazapin, Venlafaxin, Reboxetin, Moclobemid, Tranylcypromin, Bupopriion, Buspiron, Dibenzepin, Nefazodon, Opipramol, Sibutramin, and Trazodon. Most patients received only one of these antidepressants as monotherapy regiment, but some individuals received combination therapy (i.e. more than one antidepressant).

We could show a significant association of 4 SNPs in FKBP51 with response after 2 weeks of treatment.

SNP	Non-responders (n = 156)	Responders (n = 103)	p-value
rs4713916	AA:1.0%, AG:41.0%, GG:58.0%	AA:16.1%, AG:33.6%, GG:50.8%	5.5×10^{-5}
rs3800372	CC:1.0%, TC:36.9%, TT:62.1%	CC:17.9%, TC:26.3%, TT:55.8%	5.5×10^{-6}
rs1360780	TT:1.0%, CT:40.8%, CC:58.2%	TT:14.7%, CT:35.9%, CC:49.3%	1.2×10^{-4}
rs3800373	CC:0.0%, CA:37.9%, AA:62.1%	CC:13.5%, CA:33.8%, AA:52.7%	2.8×10^{-3}

Table 8: Association of SNPs in FKBP51 with response at 2 weeks.

This effect, however, was not limited to response at 2 weeks, but could be observed during the whole in-patient treatment course. This is illustrated with rs1360780 in Figure 3 and with rs3800372 in Figure 6.

All 4 SNPs genotyped in FKBP51 are in strong linkage disequilibrium (see figure 1). This implies that the causal variant could lie anywhere within the linkage disequilibrium block defined by these SNPs.

Example 3

Association of rs1360780 genotype with HPA-axis activity

To investigate changes of HPA-axis activity from admission to discharge, we used the Dex-CRH test, which sensitively identifies GR-signaling impairment¹⁸. By comparing the ACTH and cortisol responses in this test of patients with CC/CT or TT genotypes of rs1360780, we observed that at admission T homozygotes displayed a significantly lower ACTH response following CRH stimulation than patients with the CC or CT genotypes (figure 4). An elevated ACTH response in this test indicates an impaired negative feedback through GR. Our finding thus suggests that, even though T homozygotes are as severely depressed as patients with the two other rs1360780 genotypes, their GR sensitivity is not as much impaired, allowing a faster restoration of normal HPA-axis function by antidepressants and thus an accelerated clinical response.

Example 4

Association of rs1360780 genotype and FKBP51 protein expression in lymphocytes

Polymorphisms in *FKBP51* could influence GR sensitivity by several means. Polymorphisms in regulatory regions could lead to an altered expression level of

FKBP51 during the glucocorticoid stimulation of *FKBP51* and *FKBP52*

in lymphocytes. The expression of *FKBP51* and *FKBP52* was measured in lymphocytes from patients with major depressive disorder (MDD) and healthy controls (HC) at admission and discharge.

The results show that the expression of *FKBP51* and *FKBP52* was significantly lower in MDD patients at admission compared to HC and increased during treatment.

The rs1360780 genotype was associated with the expression of *FKBP51* and *FKBP52* in lymphocytes. T homozygotes showed a significantly lower expression of *FKBP51* and *FKBP52* compared to CC and CT genotypes.

could thus lead to a maintained GR sensitivity despite high circulating cortisol levels, resembling somewhat the neuroendocrine situation observed in T homozygotes. In order to investigate whether the genotype of rs1360780 was associated with different levels of FKBP51 protein, we quantified this protein in lymphocytes (T-cells) of healthy probands. Semi-quantitative analysis of Western blot results (see Figure 8) revealed that probands homozygous for the T allele of rs1360780 showed the lowest FKBP51 protein levels in lymphocytes (see Figure 9). Lower levels of FKBP51 thus could be associated with an enhanced GR sensitivity that would prevent long-lasting dysregulations of the HPA-axis (see Figure 4) and in consequence a faster response to antidepressant drugs (see Figure 3).

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Claims

1. A method of classifying an individual comprising analyzing the nucleic acid of a sample taken from said individual for nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 and/or determining the expression level of FKBP51 in said sample.
2. The method of claim 1, wherein said individual is a patient suffering from depression and said classifying consists of or comprises predicting the response to therapy.
3. The method of claim 1, wherein said classifying consists of or comprises selecting said individual for a clinical trial.
4. The method of claim 1 or 2, wherein said individual is a patient suffering from depression and said classifying consists of or comprises predicting the predisposition for an elevated number of episodes of depression.
5. The method of claim 3, further comprising the steps:
 - (a) identifying a compound modulating the activity of FKBP51; and optionally
 - (b) optimizing the pharmacological properties of the compound identified in (a); and
 - (c) performing said clinical trials;wherein said clinical trials are clinical trials of the compound identified in (a) or optimized in (b).
6. The method according to claim 5, wherein the compound modulating the activity of FKBP51 is an activator of FKBP51.
7. The method according to claim 5 or 6, wherein the compound modulating the activity of FKBP51 increases the expression of FKBP51.

8. The method of any one of claims 1 to 7, wherein the nucleic acid is gDNA.
9. The method of any one of claims 1 to 8, wherein the haplotype block comprises at least one SNP selected from the group consisting of rs4713916, rs3800372, rs1360780 and rs3800373.
10. The method of any one of claims 1 to 9, wherein the polymorphism is a SNP in a non-coding region of said haplotype block.
11. The method of any one of claims 1 to 10, wherein the polymorphism is at least one SNP selected from the group consisting of rs4713916, rs3800372, rs1360780 and rs3800373.
12. The method of any one of claims 1 to 11, wherein analyzing the nucleic acid comprises:
 - (a) a primer extension assay;
 - (b) a differential hybridization assay; and/or
 - (c) an assay which detects allele-specific enzyme cleavage.
13. The method of any one of claims 1 to 12 further comprising, prior to analyzing, amplification of at least a portion of said haplotype block.
14. The method of claim 13, wherein said amplification is effected by or said amplification is the polymerase chain reaction (PCR).
15. The method of claim 13 or 14, wherein said amplification reaction uses primers which hybridize specifically with a portion of said haplotype block.
16. The method of claim 15, wherein the primers to be used for said amplification reaction have the sequences set forth in SEQ ID NO: 1 and 2, and 3 and 4.

haplotype block which is adjacent to a polymorphism.

18. The method of claim 17, wherein the primer to be used for said primer extension assay has the sequence as set forth in SEQ ID NO: 9, 10, 11 or 12.
19. The method of any one of claims 12 to 16, wherein said differential hybridization assay or said assay detecting allele-specific enzyme cleavage uses probes which hybridize specifically with a portion of said haplotype block which comprises a polymorphism.
20. The method of claim 19, wherein said probes have the sequence as set forth in SEQ ID NO: 13 and 14; 15 and 16; 17 and 18; or 19 and 20.
21. The method of any one of claims 1 to 7, wherein the expression level to be determined is the mRNA expression level.
22. The method of any one of claims 1 to 7, wherein the expression level to be determined is the protein expression level.
23. The method of any one of claims 2, 4 or 8 to 22, further comprising the step of treating said patient suffering from depression with an antidepressant.
24. A pharmaceutical composition comprising
 - (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451;
 - (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having the biological activity of the polypeptide with the sequence of SwissProt accession number Q13451; and/or
 - (c) a polypeptide encoded by the nucleic acid of (a) or (b).
25. Use of
 - (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451;

- (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having the biological activity of the polypeptide with the sequence of SwissProt accession number Q13451;
- (c) a polypeptide encoded by the nucleic acid of (a) or (b);
- (d) an activator of the expression of the polypeptide with the sequence of SwissProt accession number Q13451; and/or
- (e) an activator of the polypeptide with the sequence of SwissProt accession number Q13451

for the manufacture of a pharmaceutical composition for the treatment of depression.

26. A method of treating depression comprising the administration of

- (a) a nucleic acid encoding a polypeptide with the sequence of SwissProt accession number Q13451;
- (b) a nucleic acid hybridizing to the complementary strand of the nucleic acid of (a) and encoding a polypeptide having the biological activity of the polypeptide with the sequence of SwissProt accession number Q13451;
- (c) a polypeptide encoded by the nucleic acid of (a) or (b);
- (d) an activator of the expression of the polypeptide with the sequence of SwissProt accession number Q13451; and/or
- (e) an activator of the polypeptide with the sequence of SwissProt accession number Q13451

to a patient suffering from depression.

27. Use of

- (a) geldanamycin or a geldanamycin derivative that can disrupt and inhibit the association between FKBP52 and Hsp90; and/or

28. A method of treating depression comprising the administration of
- (a) geldanamycin or a geldanamycin derivative that can disrupt and inhibit the association between FKBP52 and Hsp90; and/or
 - (b) an antibody or an aptamer specifically recognizing FKBP52 or a fragment or epitope thereof
- to a patient suffering from depression.

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Abstract

The present invention relates to a method of classifying an individual comprising analyzing the nucleic acid of a sample taken from said patient for nucleotide polymorphisms in a haplotype block comprising the gene encoding FKBP51 and/or determining the expression level of FKBP51 in said sample. In a preferred embodiment, the invention provides a method of predicting the response to antidepressant therapy. Furthermore, a method of developing an anti-depressant drug, and pharmaceutical compositions are provided.

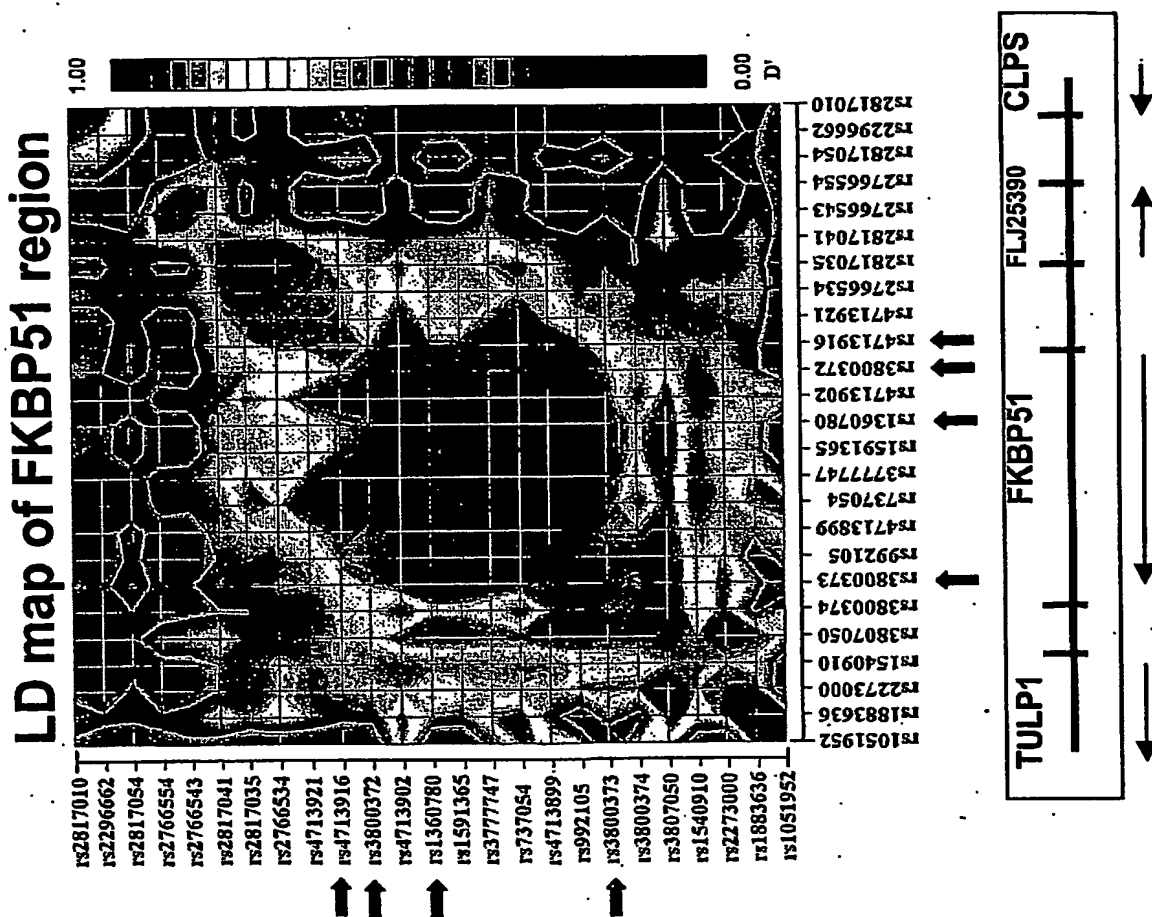
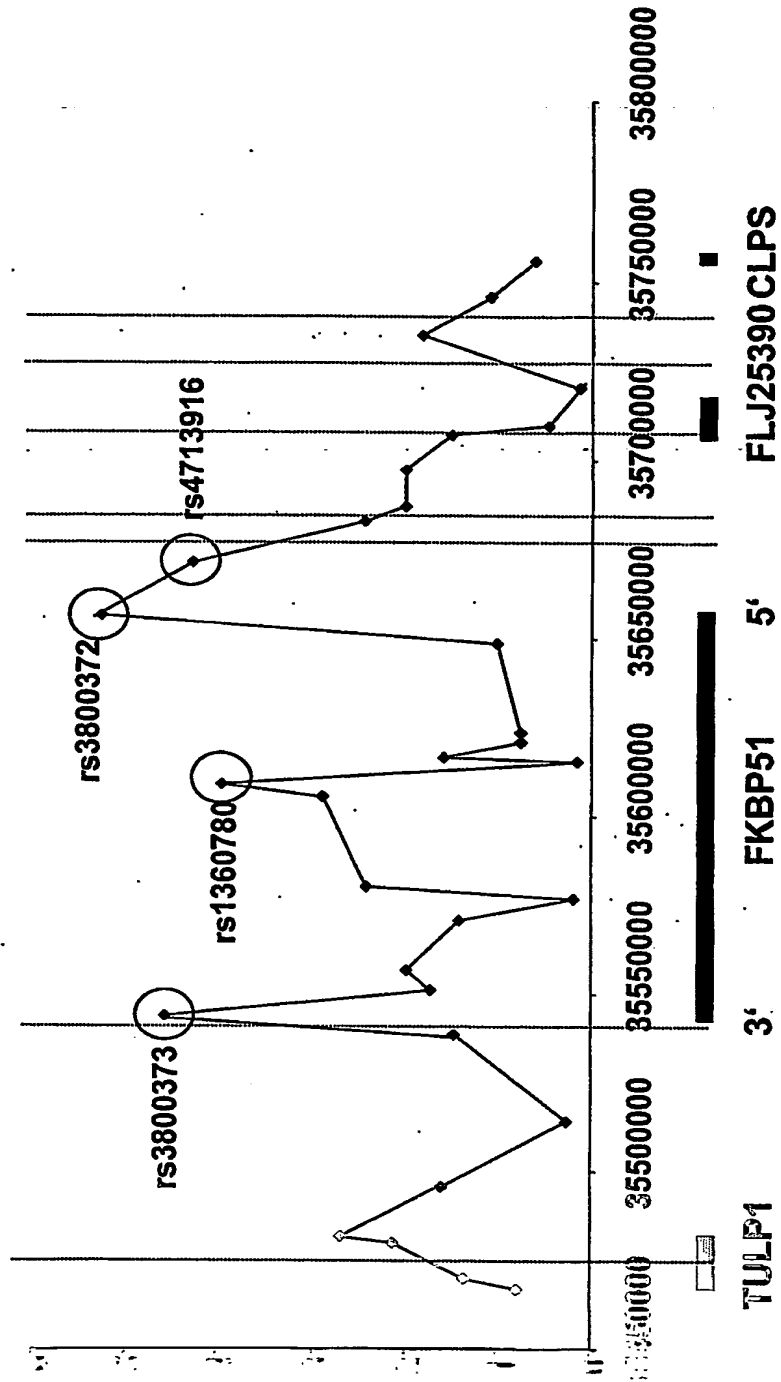


Figure 1

Association with response at 2 weeks



Association of rs1360780 SNP with antidepressant response

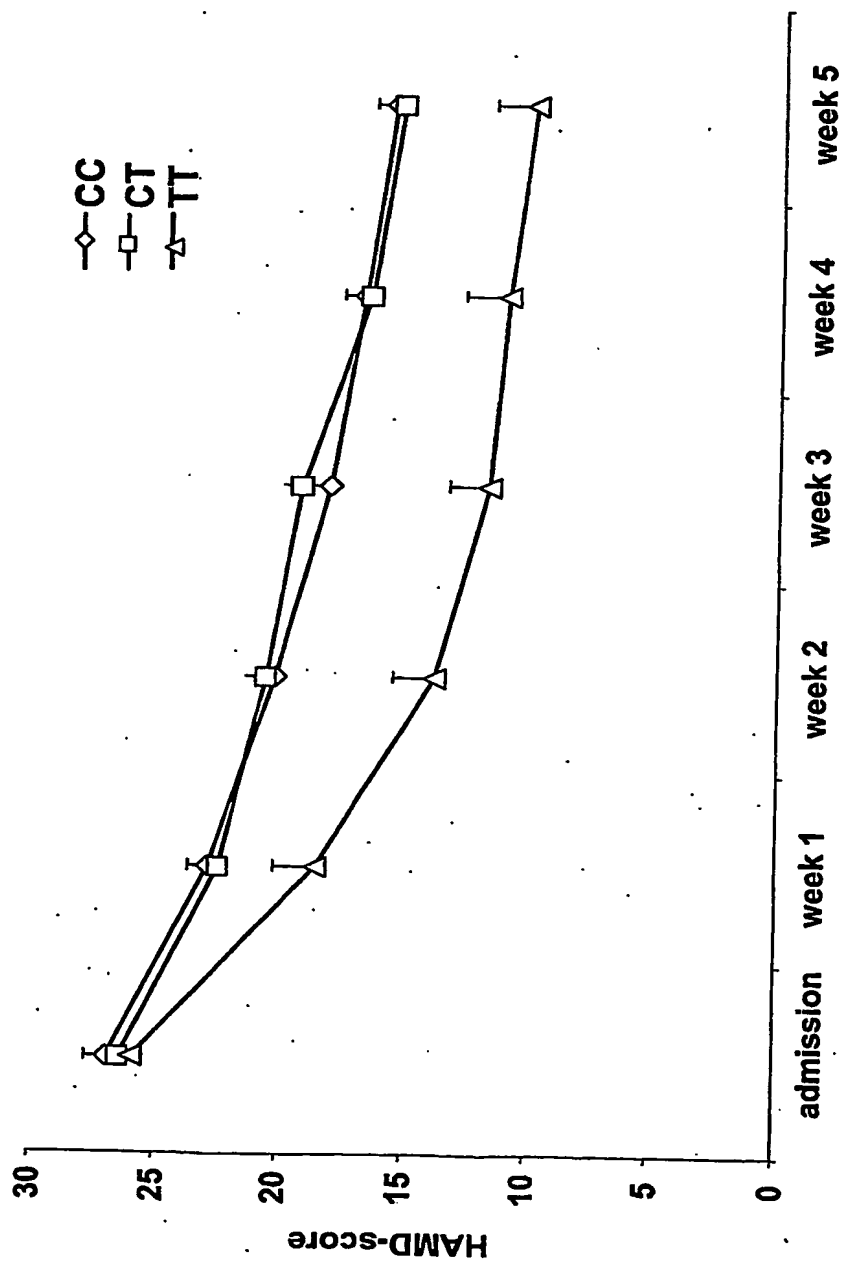
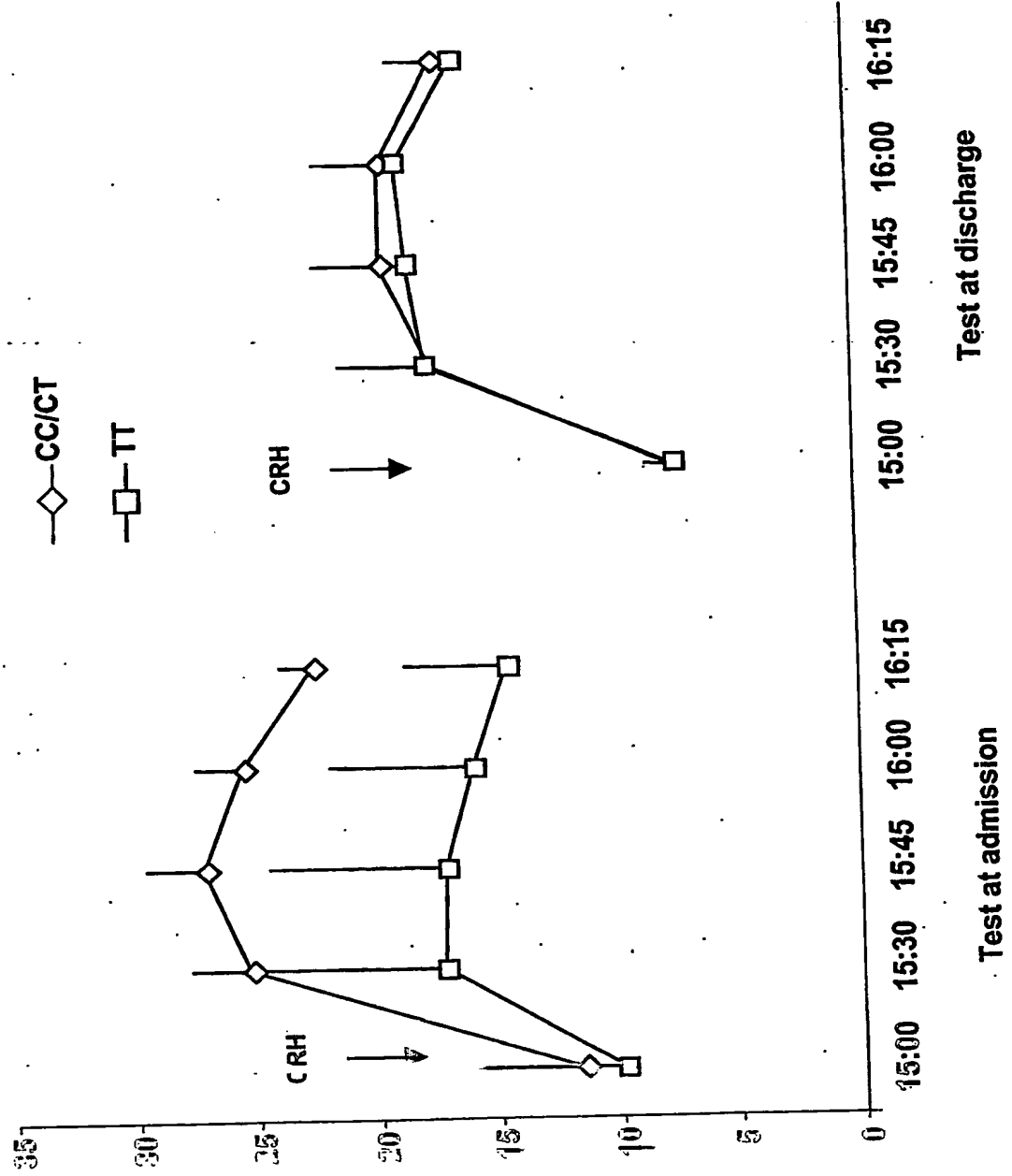


Figure 3



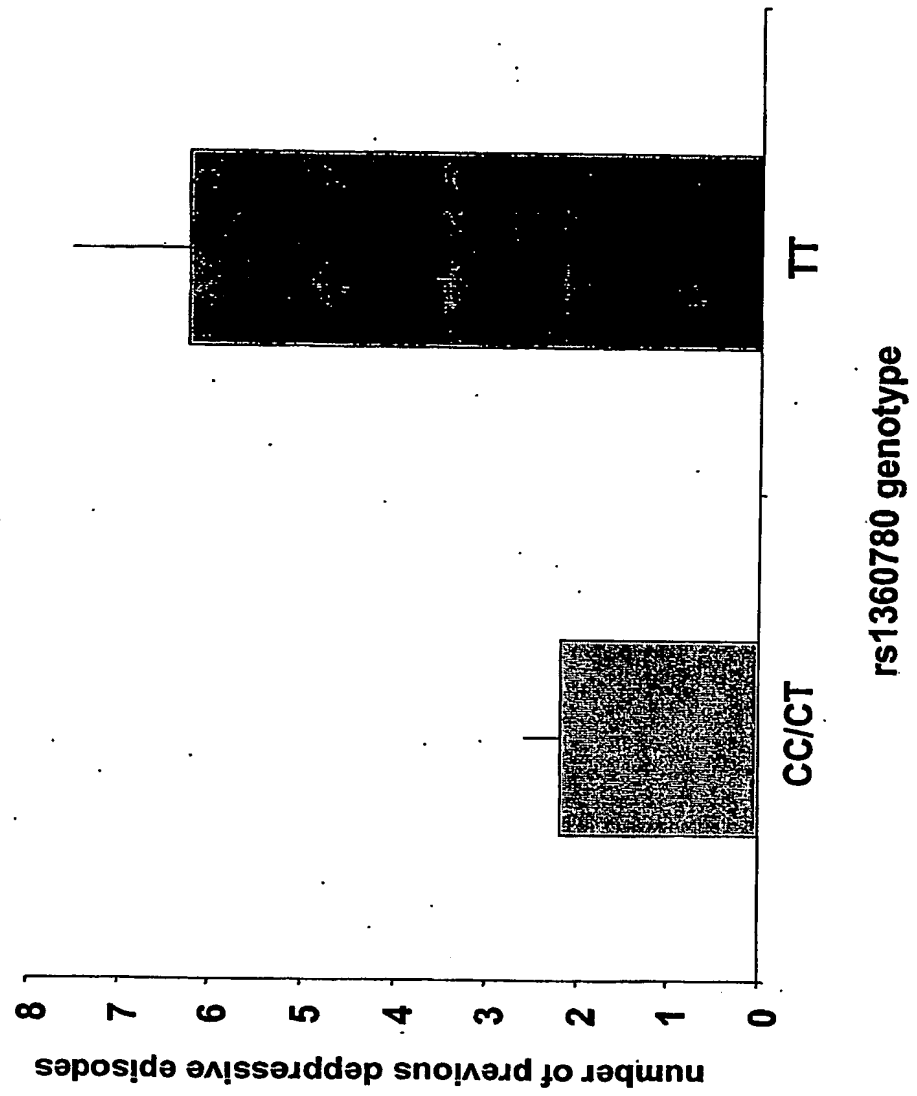
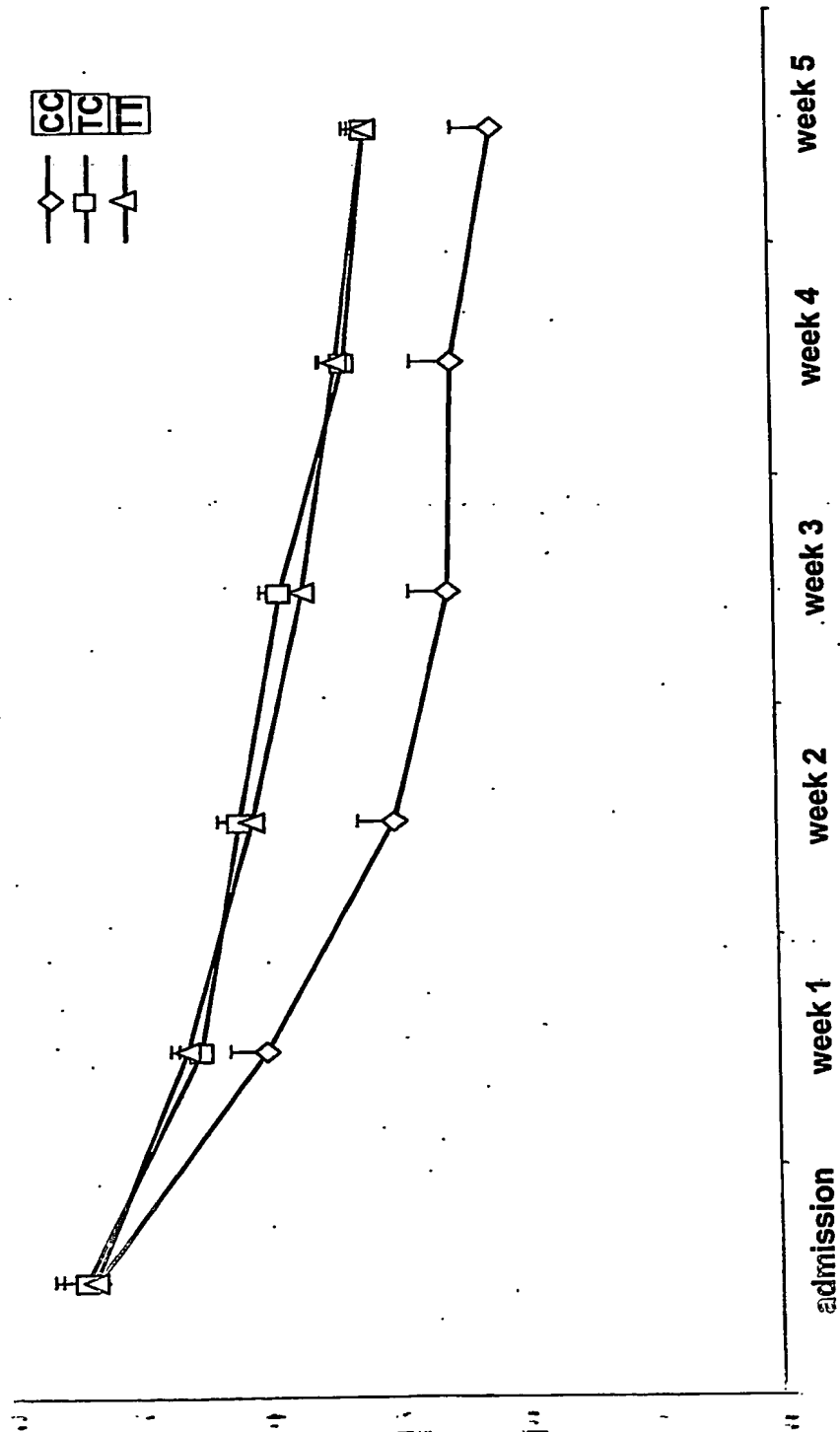


Figure 5

Association of rs 3800372 with response



Association of rs3800372 with number of depressive episodes

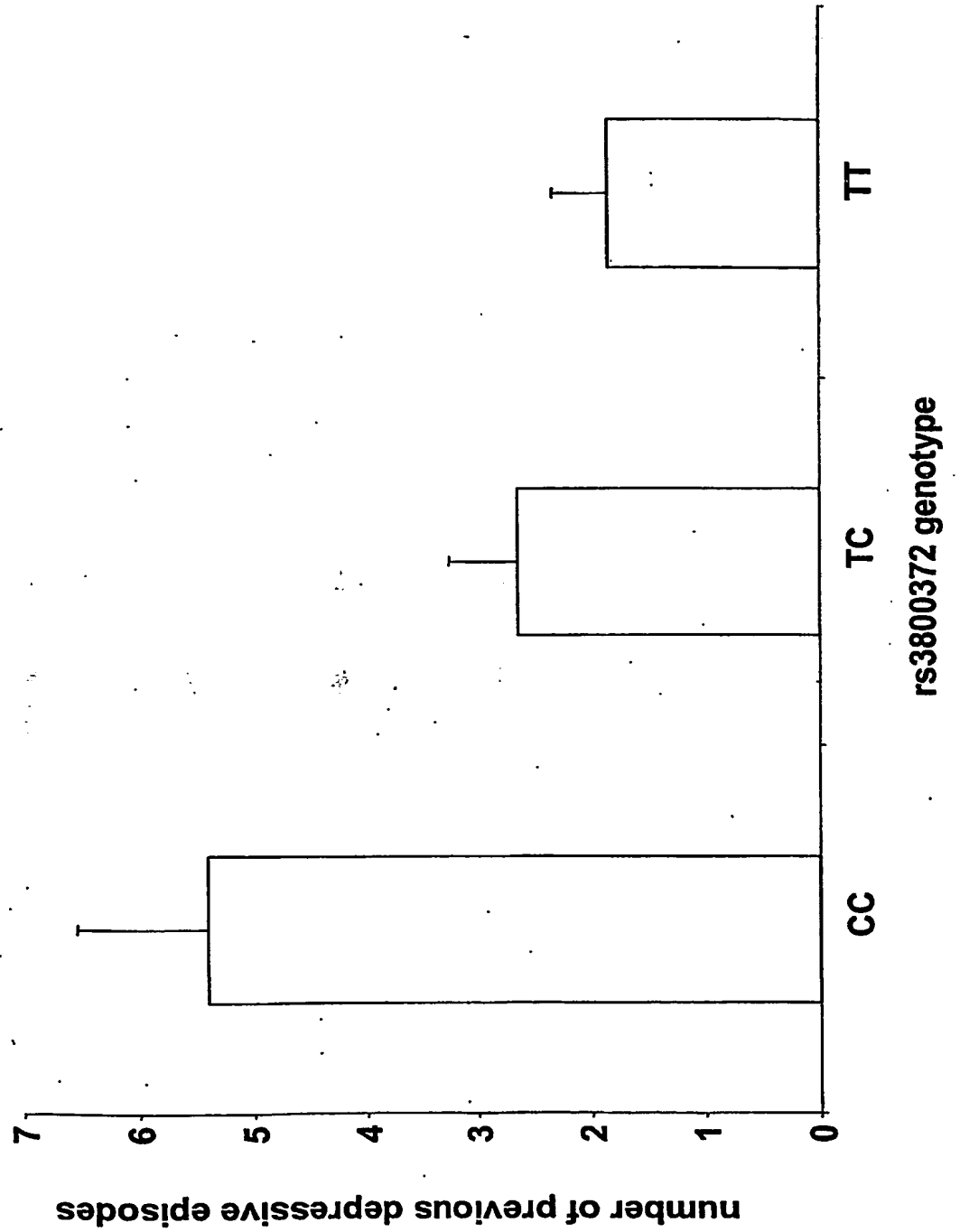


Figure 7

FKBP51 protein levels for rs3800372 SNP



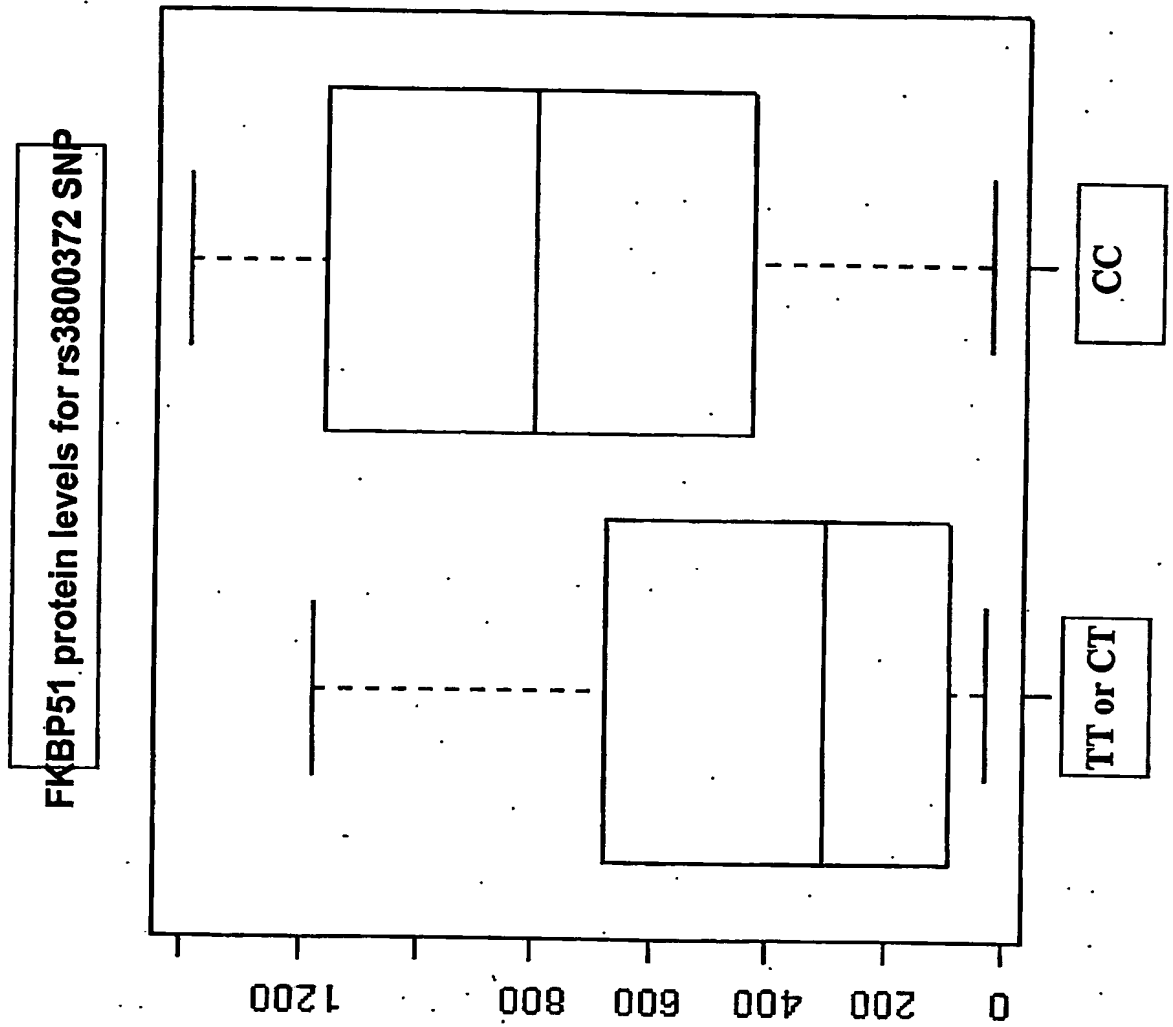


Figure 9

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